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S-acylation in plants – an expanding field

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Abstract
S-acylation is a common yet poorly understood fatty acid based post-translational modification of proteins in all eukaryotes, including plants. While exact roles for S-acylation in protein function are largely unknown the reversibility of S-acylation indicates that it is likely able to play a regulatory role. As more studies reveal the roles of S-acylation within the cell it is becoming apparent that how S-acylation affects proteins is conceptually different to other reversible modifications such as phosphorylation or ubiquitination; a new mind-set is therefore required to fully integrate these data into our knowledge of plant biology. This review aims to highlight recent advances made in the function and enzymology of S-acylation in plants, highlights current and emerging technologies for its study and suggests future avenues for investigation.

Introduction - A short history of S-acylation research in plants
While protein S-acylation has been postulated in plants since 1987 when the D1 chloroplast protein was shown to undergo reversible labelling with tritiated palmitic acid [1], it is only recently that serious progress has been made in understanding the mechanics, prevalence and effects of S-acylation. In 2005 the first S-acylating enzyme from plants was described in Arabidopsis [2], while simple means to analyse the S-acylation state in plants were defined in 2008 [3], and efforts to shed light on the Angiosperm S-acyl proteomes coming first in Arabidopsis in 2013 [4] and more recently in Poplar [5]. With these, and associated findings, S-acylation in plants has emerged as a bona fide research field able to stand alongside phosphorylation, ubiquitylation or sumoylation as an abundant post-translational modification with the potential to have major effects on protein and cellular function. A number of recent and comprehensive reviews on the topic of S-acylation in plants are available [6-8], this review will therefore focus on more recent findings while discussing concepts, future avenues for research and means of investigation.

The emerging enzymology of S-acylation in plants
S-acylation is a rapidly reversible and cycling post-translational modification yet understanding of how regulation of these processes occurs in plants is still emerging.

Protein S-acyl transferases, or PATs, are integral membrane proteins that catalyse addition of fatty acyl groups to cysteine residues within proteins (Figure 1A, B) and are characterised by an Asp-His-His-Cys (DHHC) motif [9]. The sequence of PATs is highly conserved between plants and animals and the recent elucidation of the human PAT DHHC20 structure has provided many insights into how PATs function [10] and should be applicable to plant PATs. PAT proteins have been found in all plant genomes examined to date [11]. Many of the 24 Arabidopsis (*Arabidopsis thaliana*) PATs characterised have wide

ranging but distinct phenotypes when knocked out [2, 12-17] suggesting that each enzyme has multiple substrates involved in diverse and possibly unrelated processes, but that PATs probably do not overlap to a great degree in substrate specificity. Whether this is due to inherent substrate binding specificity or potentially a result of expression pattern, sub-cellular and sub-membrane localisation is still not entirely clear. Recent work in Maize (*Zea mays*) has identified natural variation in the expression levels of the ZmTIP1 PAT as being associated with drought tolerance. Similarly to Arabidopsis *PAT24*$^{tip1-}$ mutants [2] *ZmTIP1*$^{-}$ mutants have disrupted root hair morphology and maize root hair length is positively correlated with ZmTIP1 expression levels. This suggests that ZmTIP1 mediated S-acylation affects root hair growth and, given the role of root hairs in water uptake, may explain why *ZmTIP1* high expressers have increased drought tolerance [18]. Another recent study has identified a role for Pear (*Pyrus betulifolia*) PAT14 in maintaining growth as CRISPR-Cas9 knock-out transgenic plants showed a yellow dwarf phenotype, likely caused by the reported elevated ABA levels [19].

If estimates of the numbers of S-acylated proteins per plant diploid genome is correct (between 1000-2500) then there could be upwards of 100 substrates per PAT [4]. While underlining the importance of S-acylation in plant biology this makes studying the effects of a PAT on a particular process or protein very difficult using knockouts of PATs, and may explain why few PAT-substrate pairs have been convincingly demonstrated to date using this approach. Despite this, a few PAT-substrate pairing have been proposed using PAT knock-outs, including PAT10 acting on the calcium sensor CBL10 (CalcineurinB-like 10) at the tonoplast to regulate salinity tolerance [20], PAT4 promoting ROP2 (Rho of plants 2) small GTPase membrane association [21] and PAT13/14 affecting NOA1 (NO associated 1) involved in regulating nitric oxide signalling. However, in this latter case the effect of the *pat13/14* mutation is weak and NOA1 S-acylation is not abolished [13] and PAT13 and 14 may therefore be acting redundantly with other PATs or indirectly on NOA1.

Regulatory removal of S-acyl groups is likely performed by acyl-protein thioesterases (APTs, Figure 1B) able to cleave the thioester linkage between the fatty acid and cysteine sulfhydryl. In fungi and animals 1-2 serine hydrolases (APT1/2) likely evolved from lysophospholipase A2 activities and catalyse de-S-acylation of proteins [22]. In addition, animals have another group of de-S-acylation enzymes (ABHD17A/B/C – ABhydrolase domain 17A/B/C), unrelated to APT1/2, that also act as de-S-acylating enzymes [23]. While plants contain weak sequence homologues to APT1/2 they do not contain apparent ABHD17-like proteins. This suggests that plants may harbour other de-S-acylating enzymes distinct from animals and fungi. Two recent studies suggest possible candidates for de-S-acylating enzymes in plants. In *Medicago falcata* a proposed S-acylated NAC family transcription factor is de-S-acylated in response to drought stress. The de-S-acylation is suggested to occur through the action of an acyl-ACP thioesterase from the Hotdog fold superfamily of thioesterases [24]. If sufficient biochemical evidence is found to support these initial findings it will be the first S-acyl protein thioesterase to be described in any organism outside of the serine hydrolase superfamily. As all plants possess homologues of these proposed S-acyl protein thioesterases it will be interesting to see whether this novel de-S-acylation activity is confined to *Medicago* or is found in the wider plant kingdom. Another recent paper proposes Maize ZmB6T1C9 as an S-acyl protein thioesterases [25]. However, these data are based solely on structural homology to human acyl-protein thioesterases 2 (APT2) and a 200-fold binding affinity increase for palmitate over acetate. Further biological and biochemical data on the effects of ZmB6T1C9 and homologues on S-acylated proteins are therefore required to test this hypothesis before conclusions can be drawn.

Recent insights into S-acylation in plants

Although proteomics and bioinformatics suggests that over 10% of the proteome may be S-acylated, corresponding to 40% of the membrane proteome [4], comparatively little is known about the means by which S-acylation may affect protein function, with the majority of reports highlighting its well-known but critical role as a membrane anchor for otherwise soluble proteins (e.g. [26, 27]). However, as half of all proposed S-acylated proteins contain one or more transmembrane domains [4, 5], it is unlikely that S-acylation is acting as a membrane anchor in these cases.

The cellulose synthase complex responsible for synthesising cellulose is thought to comprise a hetero-18 or -24mer made from three different CesA (cellulose synthase A) family proteins [28]. All CesA proteins contain 8 transmembrane domains and all have been found to be S-acylated, with varying numbers of S-acyl groups proposed depending on the CesA isoform in question. Mutation of the S-acylation sites in just one of the three subunits in the complex appears to render the whole complex non-functional. Quite how S-acylation has such a profound effect is unclear but, while the cellulose synthase complex assembles as expected (determine by co-immunoprecipitation of subunits), evidence from microscopy suggests that it is trapped in Golgi-derived vesicles and is unable to incorporate into the plasma membrane [29]. This may indicate a role in folding with non-S-acylated, and presumably non-catalytically active, CesA being withheld from the plasma membrane through quality control mechanisms. Alternatively, S-acylation may help define the lipid composition or structure of the vesicle that may in turn recruit other factors that aid in vesicle fusion with the PM.

Some interesting work has revealed that plant viruses co-opt S-acylation to manipulate plant proteins and facilitate virus movement and/or replication. The C4 protein of two different geminiviruses have been shown to be S-acylated and S-acylation is essential for their role in virulence. Interestingly S-acylation is essential for the plasma membrane localisation of C4 proteins and enables interaction with the plasma membrane localised CLV1/BAM (Clavata1/Barely Any Meristems1) family of receptor-like kinases involved in meristem maintenance. However, the function of the C4 protein in Geminiviruses appears to be divergent, as do the reported roles of S-acylation in C4 proteins; Mungbean yellow mosaic virus C4 S-acylation is required for supressing cell-to-cell gene silencing signals [30] while Beet severe curly top virus C4 S-acylation promotes ectopic expression of the wuschel transcription factor resulting in increased cell division in infected plant tissues [31]. Members of two more, but evolutionarily distinct plant virus families, the pomoviruses and phenuiviruses, also require S-acylation for infectivity but in these cases manipulate host S-acylated proteins to promote the viral lifestyle. The TGB1 (triple gene block 1) protein of potato mop-top virus (PMTV), the type member of the pomoviruses, is required for systemic infection. TGB1 associates with the plasma membrane early in infection where it interacts with HIPP26 (Heavy metal associated Isoprenylated Plant Protein 26). HIPP26 is localised to the plasma membrane in an S-acylation dependant manner and acts as an abiotic stress sensor. It has been hypothesised that interaction of TGB1 with HIPP26 promotes de-S-acylation of HIPP26, facilitating the observed translocation of HIPP26 to the nucleus. Nuclear localisation of HIPP26 activates drought stress responses which, in vascular tissues, allow viral access to the phloem for long distance transport and systemic infection [32]. A similar outcome is achieved through a mechanistically different route by the NSvc4 movement protein of Rice stripe virus (RSV), the phenuivirus type member. NScv4 blocks *N. benthamiana* REM (Remorin) 1.1/1.2 and rice OsREM1.4 Remorin1 (REM1) orthologue S-acylation, resulting in degradation of REM1 via an autophagy pathway. REM1 acts to prevent viral cell-to cell

movement by promoting callose deposition at plasmodesmata. By blocking REM1 S-acylation early in RSV infection and increasing REM1 turnover plasmodesmata remain callose free and do not impede viral cell-to-cell movement [33]. While S-acylation of remorins is not a new phenomenon [4, 34] this work does provide more mechanistic data on the role of S-acylation in remorin biology as non-S-acylated REM1 fails to efficiently traffic from the ER to the PM and, as a result, is turned over [33]. This suggests that Remorin S-acylation is part of a combined REM1 targeting and quality control system that is co-opted and disrupted by RSV to promote infection.

In the two major proteomic studies of S-acylation performed to date the receptor-like kinase family has featured heavily [4, 5]. Work on the receptor-like cytoplasmic kinase (RLCK) sub-group has demonstrated a role for S-acylation in acting as a membrane anchor for these otherwise soluble kinases. For the SGN1 (Schengen1) RLCK S-acylation has been shown to be dynamic with cycles of S-acylation and de-S-acylation acting to maintain an asymmetric distribution of SGN1 essential for casparian band formation in the root [35]. Initial work on receptor-like kinases suggested that S-acylation at juxta-TM cysteines had a mild positive effect on signalling outputs from the Arabidopsis FLS2 (flagellin sensing 2) flagellin receptor [4], however, recent work indicates that this is likely artefactual due to the use of epitope tags that confound FLS2 function [36] and the use of overexpression constructs. A recent reappraisal of these data using untagged FLS2 constructs driven by the native promoter and expressed at wild type levels indicates that juxta-membrane S-acylation of FLS2 is entirely dispensable for function [37]. This unexpected finding prompted a bioinformatics based assessment of juxta-membrane S-acylation in receptor-like kinases across species. Intriguingly, while juxta-TM S-acylation was not conserved in FLS2 orthologues, some receptor-like kinases have juxta-TM S-acylation sites in all orthologues examined. One such example is Erecta, a receptor-like kinase only found in angiosperms, where juxta-TM S-acylation appears to be a defining feature. As the Erecta orthologue in *Amborella trichocarpa*, the most basal angiosperm with an available genome sequence, is S-acylated, it is tempting to speculate that juxta-TM S-acylation arose with the origination of Erecta from the Erecta-like clade in the last common ancestor of Angiosperms. This absolute conservation over ~200 million years of evolution suggests that Erecta juxta-TM S-acylation is in some way essential for function. The question remains however, why is Arabidopsis FLS2 S-acylated if it is apparently not required for the described function of FLS2? As cysteines are the most reactive of all amino acids their presence in solvent exposed regions of proteins is generally only tolerated if they play a functional role. One hypothesis that resolves this apparent contradiction is that as many proteins are S-acylated adjacent to transmembrane domains this is a motif that the existing S-acylation machinery in the cell is already able to recognise. If chance mutation introduces a cysteine adjacent to a receptor-like kinase transmembrane domain S-acylation could therefore act to block the cysteine and prevent further reaction with cellular constituents. If S-acylation at this site does not negatively impact upon function then juxta-TM S-acylation will persist under neutral selection. The pattern of FLS2 orthologue juxta-TM S-acylation, with multiple gain and loss events throughout angiosperm evolution, supports this hypothesis. If, on the other hand, juxta-TM S-acylation enables neofunctionalisation of a receptor or provides a selective advantage then S-acylation at this site will become fixed. This could explain the observed ubiquity within Erecta orthologues. These data suggest a model for S-acylation, and potentially other post-translational modifications, where a protein becomes S-acylated in a non-functional or stochastic manner but is subsequently selected for and S-acylation becomes a fixed feature in all descendant species. With this in mind, assessment of candidate S-acylation sites may be aided by including orthologues from more distantly related species. Confidence in the probability of S-

acylation occurring and being functional at a site in a protein could therefore be gauged on the basis of how conserved the site is over evolutionary time. Interestingly, following on from investigating juxta-TM S-acylation, this study also found that even when juxta-TM S-acylation was abolished both Erecta and FLS2 remained S-acylated, albeit at lower levels. Testing of a range of other receptor-like kinases that naturally lack juxta-TM sites indicated that they were also S-acylated and suggests that receptor-like kinases have additional S-acylation sites [37]. The location and function of these sites is currently unknown.

Current and emerging methods to study S-acylation

As S-acylation research has progressed so too have the tools available to study it. Initially the only approaches available to researchers were hydroxylamine sensitive metabolic incorporation using radioisotope labelled fatty acids or cell biology coupled with mutagenesis. Radioisotope fatty acid labelling of proteins in plants is apparently not trivial and has only been reliably reported twice [1, 38] and in neither case using intact plants. Cell biology studies using mutagenesis are much more common, but the major downside is that while mutagenesis of a cysteine may alter subcellular localisation there is no direct evidence to support S-acylation of the cysteine in question. While proteins that are solely anchored to membranes by S-acylation are amenable to study via microscopy and mutagenesis, if you are dealing with an integral membrane protein, there is no guarantee that mutation of an S-acylation site will affect subcellular distribution [37] and sub-membrane scale changes are unlikely to be visible without specialized quantitative techniques [39].

Many studies have made use of 2-bromopalmiatate to inhibit S-acylation (Figure 1B). While potent, 2-bromopalmiatate is not particularly specific to S-acylation, particularly when used for prolonged periods of time. It has also been reported to irreversibly modify authentic sites of S-acylation, potentially mimicking the process it is meant to be preventing [40]. At high concentrations or with prolonged exposure it is lethal to the cell. Care must therefore be taken in its use to ensure that observed effects for a protein are due to changes in S-acylation state rather than collapse of cellular function. Typical concentrations used for Arabidopsis are 10-50 μM for less than 12 hours [2] but these values will change depending on the process, tissue type, read out and culture method being used. Inhibition of de-S-acylation is possible in mammalian systems through the use of commercially available palmostatin B or ML211, both reported inhibitors of APT1/2 [22, 41] with palmostatin B also able to inhibit de-S-acylation mediated by ABHD17 [23]. In the authors' laboratory both of these compounds are capable of inducing mild phenotypic changes in Arabidopsis root hairs (Laurila and Hemsley, unpublished observations), but we have been unable to observe any effect on plant proteins that undergo cycles of S-acylation, suggesting that the observed phenotypic effects are not primarily due to alterations in S-acylation but may instead be due to inhibition of other enzyme activities. This may indicate that some or all plant de-S-acylating enzymes are not closely related to described mammalian enzymes, as has already been suggested above [24].

Advances in the biochemical study of S-acylation have led to many SDS-PAGE based hydroxylamine mediated S-acyl thioester cleavage assays (Figure 1A) being developed, including acyl-biotin exchange (ABE), acyl-resin assisted capture (acyl-RAC) [42] and acyl-PEG exchange (APE) [43]. Additionally hydroxylamine mediated differential labelling coupled with mass spectrometry has been used to map S-acylation sites [44]. However, a caveat with hydroxylamine based assays is their indirect nature and the fact that S-acylated proteins are not the only thioester containing protein in the cell. For example,

E2-ubiquitin ligases transfer ubiquitin via a thioester, nitrilases form thioesters as a reaction intermediate and enzymes containing phosphopantetheine groups (e.g. acyl-carrier proteins) use the terminal thiol of phosphopantetheine to form a thioester with substrates. Despite this, many of these "false positive" proteins are known and well characterised and can therefore be excluded from data sets. However, thioesters in proteins also remain relatively rare, making hydroxylamine-based assays a good, quantitative route into the study of S-acylation as they are fast and simple to perform for any molecular biology lab. At the time of writing GE Healthcare have ceased production of thiopropyl-Sepharose 6b, making Acyl-RAC assays as described in the literature [42] impossible. It is not yet known if manufacture will resume or if another manufacturer will fill the market gap.

Reports of direct detection of S-acylation on plant proteins is limited to date, with the most effective utilizing GC-MS [45, 46]. The disadvantage of this method is the quantity of plant material required for analysis. In animals an alternative route using metabolic labelling with azide or alkyne functionalised fatty acid analogues coupled with click chemistry is now standard in the field. One report suggests that metabolic labelling in Arabidopsis protoplasts can be successful [47] but these methods have yet to be taken up by the community and, based on experiences in the author's lab, are non-trivial when compared to similar experiments in animal or yeast systems. This may be due to plants being autotrophic and therefore may not produce proteins able to facilitate uptake of extracellular fatty acids in the way that animals and fungi do. Alternatively, fatty acids may not end up in the appropriate sub-cellular compartment for effective conversion to the acyl-CoAs required for S-acylation to occur or, if they are converted to acyl-CoAs, they may get diverted to other pathways such as lipid synthesis. A possible route around some of these potential issues may be to use alternative fatty acid delivery methods, such as tween-style esters of alkyne fatty acids which have been reported to efficiently deliver radiolabelled fatty acids into plant cells [48]. One development that may open the way to direct detection of S-acylated proteins is mass spectrometry, although the peptide fractionation set up required is not that typically found in proteomic facilities and the practicalities of application beyond model peptides has not been investigated [49].

As has been mentioned S-acylation is not a homogenous modification, different length and saturations of fatty acids can be added to the same protein in different proportions, at different times or on different residues [45, 50]. This chain length specificity is controlled, at least in part, by the dimensions of the fatty acid binding pocket of the PAT enzymes themselves [10]. This makes direct detection problematic as metabolic labelling may not provide the most appropriate fatty acid [50], while direct LC-MS of S-acylated peptides [49] may require a corresponding standards library of each possible S-acylated peptide to unambiguously identify a peptide with specific acyl chains. If analysis of a specific protein is being performed, and one of the rapid hydroxylamine based assays suggests that it is S-acylated, a recent innovation may help with these issues. Hydroxylamine derivatives have been developed that allow for cleavage of S-acyl groups from proteins and identification by liquid chromatography and high resolution mass spectrometry (LC-HRMS) [51]. This would inform the researcher as to which fatty acid alkynes should be used in metabolic labelling approaches or define which peptides should be used in generating a peptide library for LC-MS based proteomics. This method would also unambiguously identify the protein as S-acylated.

The future of S-acylation research in plants

While the number of groups studying S-acylation in plants is small compared to mammalian systems the levels of knowledge are similar and cross-informative. Plants will therefore continue to play a role in the study of S-acylation, particularly where whole organism, developmental or genetic approaches are required to answer a question. Finally, plants contain a great many S-acylated proteins not found in other eukaryotes [2, 4, 5, 26, 29, 32, 52, 53]; study of S-acylation in the function of these proteins is therefore impossible without studying the plants themselves.

Despite the importance and prevalence of S-acylation in plants being established there are still large gaps in our knowledge. The major questions remain thus: **1.** Which proteins undergo changes in S-acylation state in response to a stimulus or signal and which are constitutively S-acylated? **2.** What are the enzymes that remove S-acyl groups from plant proteins? **3.** How does S-acylation exert its effects on protein function? **4.** Do plastids perform S-acylation and if so is it mechanistically different to "eukaryotic" S-acylation?

Perspectives

1. S-acylation is a prevalent yet understudied reversible post-translational modification found on nearly 50% of plant membrane proteins. The functional impact of S-acylation for most of these proteins is unknown.
2. S-acylation alters protein interactions with membranes and has been described to affect subcellular and sub-membrane distribution, protein activation state, protein-protein interactions, protein turnover and protein conformation.
3. Future work on S-acylation in plants should focus on determining how S-acylation, and particularly reversible S-acylation, impacts upon function. This knowledge will increase understanding of basic cellular biology and highlight the importance of membrane protein regulation in plant cell function.

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Figures

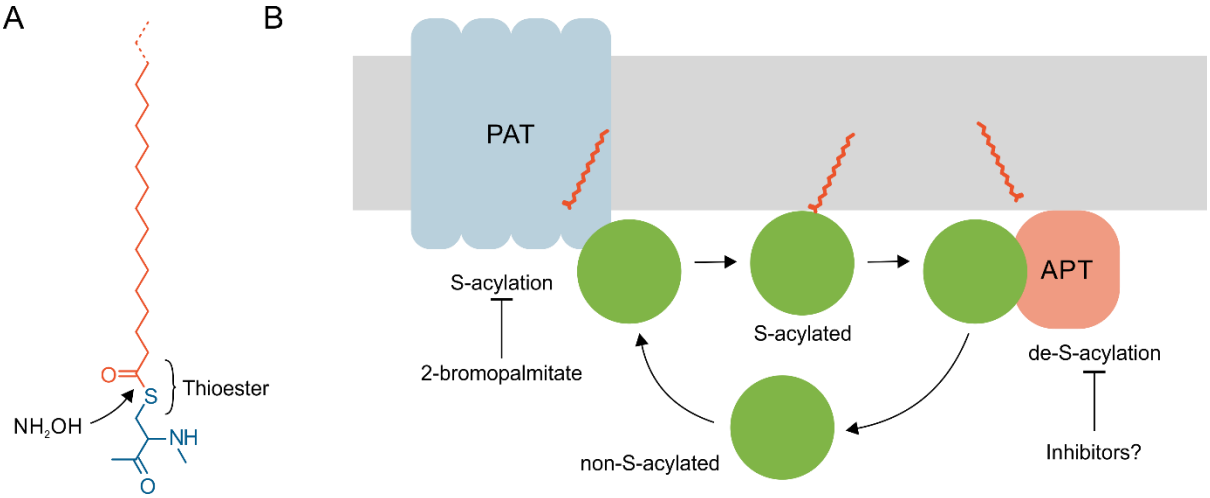

Figure 1 A. Long chain fatty acids (orange; palmitic acid (C16) shown in solid line, stearic acid (C18) additional 2 carbons shown as dashed line) are added to cysteine residues (blue) through a thioester

bond. Many assays to investigate S-acylation take advantage of hydroxylamine (NH_2OH) to cleave the thioester revealing a free sulfhydryl on the cysteine residue. **B.** Summary of mechanism and enzymology of S-acylation in plants; S-acylation is catalysed by protein S-acyltransferases (PAT) and removed by as yet unknown acyl-protein thioesterases (APTs). 2-bromopalmitate inhibits PAT action but no inhibitors of de-S-acylation have been described in plants.

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